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A new sexannulate species of *Orobdella* (Hirudinea, Arhynchobdellida, Orobdellidae) from Kii-Oshima Island, Japan

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Abstract

A large-type sexannulate leech species, *Orobdella ibukifukuyamai* **sp. nov.**, from Kii-Oshima Island, Japan, is described based on morphologic and molecular data. Phylogenetic analyses using nuclear 18S rRNA, 28S rRNA, histone H3, mitochondrial cytochrome *c* oxidase subunit I, $tRNA^{Cys}$, $tRNA^{Met}$, 12S rRNA, $tRNA^{Val}$, 16S rRNA, $tRNA^{Leu}$ and NADH dehydrogenase subunit 1 markers showed that *O. ibukifukuyamai* formed a clade with the sexannulate *O. okanoi* Nakano, 2016 and *O. yamaneae* Nakano, 2016 and octannulate *O. nakahamai* Nakano, 2016, which are endemic to Shikoku island, Japan.

Key Words

Hirudinida, Erpobdelliformes, Japanese Archipelago, molecular phylogeny

Introduction

Leeches that belong to the gastroporous genus *Orobdella* Oka, 1895 are indigenous to terrestrial habitats in Far East Asia (Sawyer 1986; Nakano 2017a), and are classified into 22 species (Nakano 2021). The species are grouped into three morphotypes by the mid-body somite annulation, i.e., 4- (quadr-), 6- (sex-), and 8- (oct-) annulate, and can also be categorized into three morphotypes by the body lengths of mature individuals, i.e., small (~5 cm), middle (~10 cm), and large (~20 cm) types (Nakano 2017a). All of the six sexannulate and two octannulate *Orobdella* leeches are denoted as the middle- or large-type species, and endemic only to the Japanese Archipelago, including the Ryukyu Islands (Nakano 2017a). Previous molecular phylogenetic analyses revealed that sexannulation and octannulation, respectively, have evolved in parallel within the genus *Orobdella* (Nakano 2016).

Unidentified large-type sexannulate individuals of *Orobdella* were collected from Kii-Oshima Island, which is located near the southern tip of the Kii Peninsula of Honshu island, Japan, and they are herein described as a new species. In addition, its phylogenetic position is investigated using nuclear and mitochondrial genetic markers.

Materials and methods

Samples and morphological examination

Two leeches were collected from a small scree slope on Kii-Oshima Island, Japan. Specimens were relaxed by the gradual addition of absolute ethanol (EtOH) to fresh water, and then fixed in absolute EtOH. For DNA extraction, botryoidal tissue was removed from the posterior part around the caudal sucker of each specimen, and then preserved in absolute EtOH. The remainder of the body was fixed in 10% formalin, and preserved in 70% EtOH. Four measurements were taken: body length from the anterior margin of the oral sucker to the posterior margin of the caudal sucker, maximum body width, caudal sucker length from the anterior to the posterior margin of the sucker, and caudal sucker width from the right to the left margin of the sucker. Examination, dissection, and draw-

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ing of the specimens were conducted using a stereoscopic microscope with a drawing tube (M125, Leica Microsystems, Wetzlar, Germany). Specimens examined in this study have been deposited in the Zoological Collection of Kyoto University (**KUZ**).

The numbering convention is based on Moore (1927): body somites are denoted by Roman numerals, and the annuli in each somite are given alphanumeric designations. The following abbreviations are using for morphological characters in the text and figures: **ac** atrial cornu; **af** annular furrow; **an** anus; **at** atrium; **BL** body length; **BW** body width; **CL** caudal sucker length; **cod** common oviduct; **cp** crop; **CW** caudal sucker width; **ed** ejaculatory duct; **ep** epididymis; **fg** female gonopore; **gd** gastroporal duct; **gp** gastropore; **mg** male gonopore; **np** nephridiopore; **od** oviduct; **ov** ovisac; **ph** pharynx; **ts** testisac.

Molecular analyses

The phylogenetic position of the new *Orobdella* species within the genus was investigated based on three nuclear and three mitochondrial markers, i.e., 18S rRNA, 28S rRNA, histone H3, cytochrome *c* oxidase subunit I (COI), $tRNA^{Cys}$, $tRNA^{Met}$, 12S rRNA, $tRNA^{Val}$, and 16S rRNA ($tRNA^{Cys}$–16S) and $tRNA^{Leu}$ and NADH dehydrogenase subunit 1 ($tRNA^{Leu}$–ND1). Methods for the genomic DNA extraction, polymerase chain reactions and cycle sequencing reactions were elucidated in (Nakano and Lai 2016, 2017). In total, nine sequences were newly obtained and deposited with the International Nucleotide Sequence Databases (**INSD**) through the DNA Data Bank of Japan (Suppl. material 1: Table S1).

In addition to the newly obtained sequences, 181 sequences of 22 *Orobdella* species and eight erpobdelliform taxonomic units, which were selected as the outgroup, were obtained from the INSD in accordance with the previous studies (Nakano 2021), and were included in the present dataset (Suppl. material 1: Table S1). The alignments of H3 and COI were trivial, as no indels were observed. The sequences of the other markers were aligned using MAFFT v. 7.471 L-INS-i (Katoh and Standley 2013). The lengths of the 18S, 28S, H3, COI, $tRNA^{Cys}$–16S, and $tRNA^{Leu}$–ND1 were 1850, 2823, 328, 1267, 1182, 640 bp, respectively. The concatenated sequences thus yielded 8090 bp of aligned positions.

Phylogenetic trees were reconstructed using maximum likelihood (ML) and Bayesian inference (BI). The best-fit partition scheme and models were identified based on the Bayesian information criterion using PartitionFinder v. 2.1.1 (Lanfear et al. 2017) with the 'greedy' algorithm (Lanfear et al. 2012). The selected partition scheme and models were as follows: for 18S and H3 1st position, K80+I+G; for 28S, GTR+I+G; for H3 2nd position, JC+I; for H3 3rd position, GTR+G; for COI 1st position, GTR+G; for 2nd positions of COI and ND1, GTR+I+G for BI; for 3rd positions of COI and ND1, KHY+G; for $tRNA^{Met}$ and ND1 1st position, GTR+I+G; for $tRNA^{Cys}$, 12S, $tRNA^{Val}$ and $tRNA^{Leu}$, GTR+I+G; and for 16S, GTR+G. The ML tree was calculated using IQ-Tree v. 2.1.3 (Minh et al. 2020) with non-parametric bootstrapping (BS) conducted with 1000 replicates. BI tree and Bayesian posterior probabilities (PP) were estimated using MrBayes v. 3.2.7a (Ronquist et al. 2012). Two independent runs for four Markov chains were conducted from 40 million generations, and the tree was sampled every 100 generations. The parameter estimates and convergence were checked using Tracer v. 1.7.1 (Rambaut et al. 2018), and then first 100 001 trees were discarded based on the results.

Pairwise comparisons of uncorrected *p*-distances for COI (1267 bp) sequences of the newly identified *Orobdella* and its close congeners were calculated using MEGAX (Kumar et al. 2018).

Taxonomy

Family Orobdellidae Nakano, Ramlah & Hikida, 2012

Genus *Orobdella* Oka, 1895

Type species. *Orobdella whitmani* Oka, 1895, fixed by subsequent designation (Soós 1966).

***Orobdella ibukifukuyamai* sp. nov.**

https://zoobank.org/824E7A4A-7FBC-49D7-B457-3EE3F40BBABA

Figs 1–3

Diagnosis. Body length of mature individuals reaching ~15 cm. Somite VII quinquannulate, somites VIII–XXV sexannulate, b1 = b2 < a2 = b5 > c11 = c12 (especially mid-body somites). Male gonopore in slightly anterior to middle of, or in middle of somite XI c12, female gonopore in somite XIII b2/a2, posterior to gastropore, gonopores separated by 1/2 + 8 annuli. Pharynx reaching to somite XIV a2/b5–b5. Gastropore conspicuous, in

Figure 1. *Orobdella ibukifukuyamai* sp. nov., holotype, KUZ Z4031. **A.** Dorsal view; **B.** Ventral view; **C.** Dorsal view of live animal. Scale bars: 20 mm (same bar for **A, B**).

somite XIII b2/a2. Gastroporal duct thick tubular. Paired epididymides in somites XVI–XVIII, occupying 11–12 annuli. Atrial cornua developed, ovate, without pre-atrial loop. Dorsal surface reddish brown in life.

Material examined. ***Holotype*: Japan** • Wakayama Prefecture, Kushimoto Town, Kii-Oshima Island, near Minato Shrine; 33°28.3'N, 135°48.3'E; 20 Nov. 2021; I. Fukuyama leg.; KUZ Z4031. ***Paratype*: Japan** • same data as for holotype; KUZ Z4032.

Description (based on the holotype KUZ Z4031). Body firm and muscular, elongate, with constant width in caudal direction, dorsoventrally compressed, BL 149.5 mm, BW 8.9 mm (Fig. 1A, B). Caudal sucker ventral, almost circular, CL 4.4 mm, CW 4.9 mm (Figs 1B, 2C, D).

Somite I completely merged with prostomium. Somite II (= peristomium) uniannulate (Fig. 2A) Somites III–V biannulate (Fig. 2A): III and IV, (a1 + a2) > a3; V, (a1 + a2) = a3, a3 forming posterior margin of oral sucker (Fig. 2A, B). Somite VI triannulate, a1 (with obvious secondary furrow dorsally, b1 = b2) > a2 = a3 (Fig. 2A, B). Somite VII quinquannulate, b1 = b2 = a2 = b5 = b6 (Fig. 2A, B). Somites VIII–XXV sexannulate (Fig. 2A–E): VIII–XII, b1 = b2 = a2 = b5 = c11 = c12; XIII b1 = b2 < a2 > b5 = c11 = c12; XIV and XV, b1 = b2 = a2 = b5 = c11 = c12; XVI–XVIII, b1 < b2 = a2 (b3 = b4 dorsally) = b5 > c11 = c12 (Fig. 2E); XIX–XXII, b1 < b2 (c3 = c4 dorsally) = a2 (b3 = b4 dorsally) = b5 > c11 = c12; XXIII and XXIV, b1 < b2 = a2 (b3 = b4 dorsally) = b5 > c11 = c12; XXV and XXVI, b1 = b2 = a2 = b5 = c11 = c12. Somite XXVII dorsally quadrannulate, b1 = b2 = a2 < a3; b1 being ventrally last complete annulus (Fig. 2C, D). Anus posterior to somite XXVII; post-anal annulus absent (Fig. 2C).

Male gonopore slightly anterior to middle of somite XI c12 (Fig. 2F). Female gonopore in somite XIII b2/a2, inconspicuous, located posterior to gastropore (Fig. 2F, G). Gonopores separated by 1/2 + 8 annuli (Fig. 2F).

Anterior ganglionic mass in somite VI a2 and a3. Ganglion VII in somite VII b2. Ganglia VIII and IX, of each somite, in b2 and a2. Ganglia X and XI, of each somite, in a2 (Fig. 3B). Ganglia XII and XIII, of each somite, in b2 and a2 (Fig. 3B). Ganglion XIV in somite XIV a2 (Fig. 3B). Ganglion XV in somite XV b2 and a2 (Fig. 3B). Ganglion XVI in somite XVI a2 (Fig. 3B). Ganglia XVII–XXII, of each somite, in b2 and a2 (Fig. 3B). Ganglia XXIII and XXIV, of each somite, in a2. Ganglion XXV in somite XXV b2 and a2. Ganglion XXVI in somite XXVI b1 and b2. Posterior ganglionic mass in somite XXVI c11 and c12.

Eyespots in 3 pairs, 1st pair dorsally on somite II/III (Fig. 2A), 2nd and 3rd pairs dorsolaterally on posterior margin of somite V (a1 + a2). Papillae numerous, minute, hardly visible, 1 row on dorsal surface of every annulus.

Nephridiopores in 17 pairs, each situated ventrally at posterior margin of b2 of each somite in somites VIII–XXIV (Fig. 2B, D, F).

Pharynx agnathous, euthylaematous, reaching to somite XIV a2/b5 (Fig. 3A). Crop tubular, acecate, reaching to somite XXI c11/c12. Intestine tubular, acecate, reaching to somite XXV b1/b2. Rectum tubular, thin-walled, straight. Gastropore conspicuous, ventral, in somite XIII b2/a2 (Fig. 2F, G). Gastroporal duct thick, tubular, slightly winding at junction with gastropore, reaching to somite XIV a2 and connecting with anteriormost part of crop (Fig. 3A).

Testisacs multiple in somite XIX b1 to XXV c12 (Fig. 3B); on right side, in total ~83 testisacs, 13 in XIX, 14 in XX, 13 in XXI, 11 in XXII, 12 in XXIII, 11 in XXIV, 9 in XXV; on left side, in total ~80 testisacs, 12 in XIX, 12 in XX, 13 in XXI, 14 in XXII, 11 in XXIII, 10 in XXIV, 8 in XXV. Paired epididymides; right epididymis in somite XVII b1 to somite XVIII/XIX, occupying 12 annuli; left epididymis in somite XVII b1/b2 to somite XVIII/XIX, occupying 11 annuli (Fig. 3B). Paired ejaculatory ducts; right duct in somite XI b5 to somite XVII b1; left duct in somite XI b5 to somite XVII b1/b2; coiled in position posterior to ovisacs; each duct crossing ventrally beneath each ovisac, then nearly straight in position anterior to ovisacs; each widening from respective junction with epididymis, narrowing at junction with atrial cornua, then turning proximally toward atrial cornua without pre-atrial loop (Fig. 3B, C). Pair of muscular atrial cornua developed, ovate, in somite XI b5–c12 (Fig. 3B–E). Atrium short, muscular, globular in somite XI c11 and c12 (Fig. 3C–E).

Paired ovisacs globular, in somite XIII a2 and b5 (Fig. 3B, F). Oviducts thin-walled, left oviduct crossing ventrally beneath nerve cord (Fig. 3B, F); both oviducts converging into common oviduct in somite XIII a2. Common oviduct thin-walled, short, directly descending to female gonopore (Fig. 3F).

Coloration (based on the holotype KUZ Z4031 and paratype KUZ Z4032). In life, dorsal surface reddish brown (Fig. 1C); lateral surface ocher; ventral surface whitish yellow. Color faded in preservative; dark mid-dorsal line present from somite VII b6 to somite XXVII only in paratype.

Variation (based on the paratype KUZ Z4032). Measurements: BL 121.1 mm, BW 6.3 mm, CL 3.3 mm, CW 4.1 mm. Somites XVI–XXI sexannulate, b1 = b2 < a2 (b3 = b4 dorsally) = b5 (c9 = c10 dorsally) > c11 > c12. Pharynx reaching to somite XIV b5. Crop reaching to somite XXI/XXII. Gastroporal duct reaching to somite XIV a2/b5. Intestine reaching to somite XXV b1. Paired epididymides in somite XVI b2/a2 to somite XVIII b1/b2, occupying 11 annuli. Paired ejaculatory ducts in somite XI b5 to somite XVI b2/a2; coiled in position anterior to ovisacs. Paired ovisacs; right ovisac in somite XIII a2–c11; left ovisac in somite XIII a2 and b5. Right oviduct crossing ventrally beneath nerve cord.

Etymology. The specific name is dedicated to Mr Ibuki Fukuyama who collected the specimens of the new species.

Distribution. This species was collected only from the type locality.

Molecular phylogenetic position and genetic distances. The ML (Fig. 4) and BI (not shown) trees had identical topologies, and were explicitly concordant with those of previous analyses (e.g., Nakano 2021). *Orobdella ibukifukuyamai* was placed within the clade that consists of the sexannulate *O. yamaneae* Nakano, 2016 and *O. okanoi*

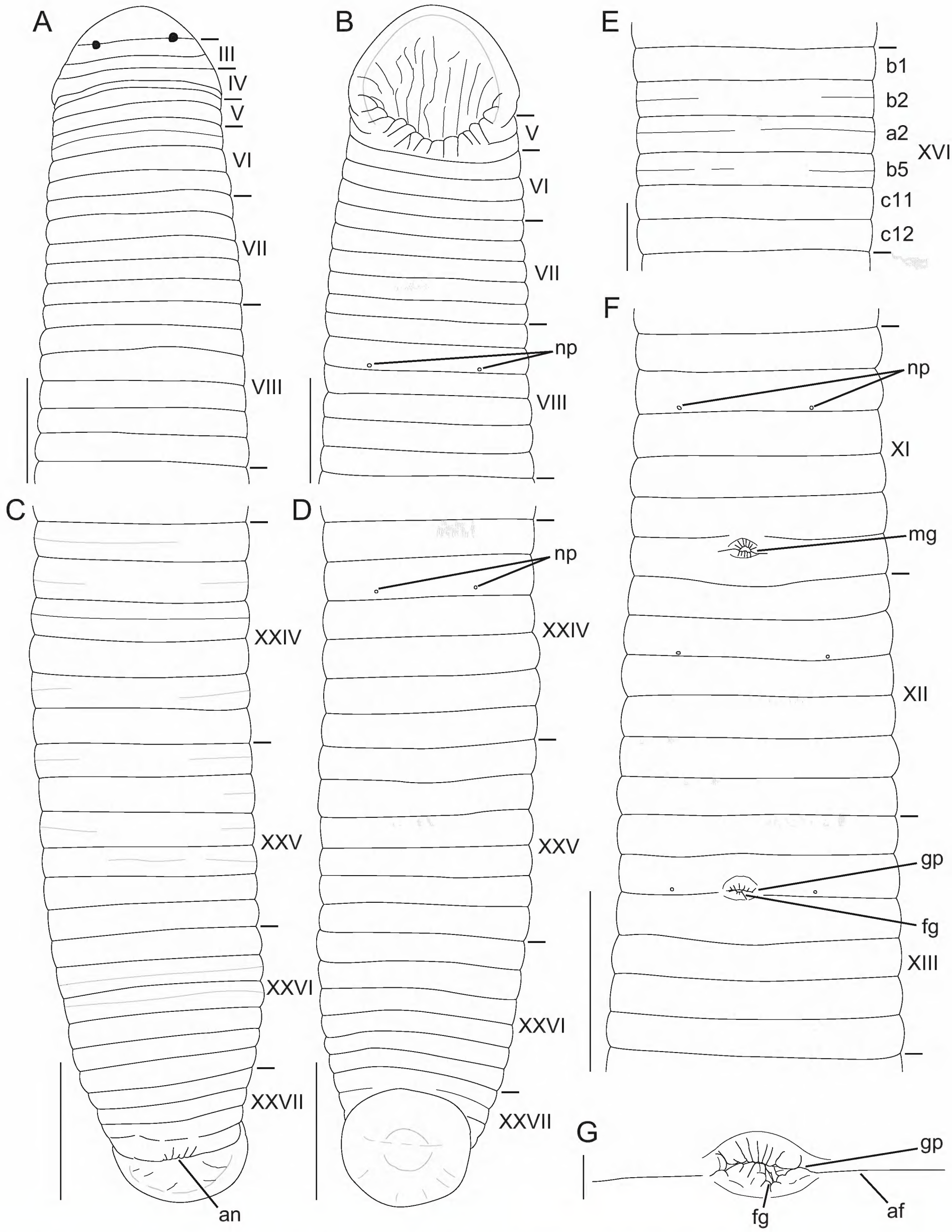

Figure 2. *Orobdella ibukifukuyamai* sp. nov., holotype, KUZ Z4031. **A.** Dorsal view of somites I–VIII; **B.** Ventral view of somites I–VIII; **C.** Dorsal view of somites XXIV–XXVII and caudal sucker; **D.** Ventral view of somites XXIV–XXVII and caudal sucker; **E.** Dorsal view of somite XVI; **F.** Ventral view of somites XI–XIII; **G.** Ventral view of gastropore and female gonopore. Scale bars: 2.5 mm (**A, B, E**); 5 mm (**C, D, F**); 0.5 mm (**G**).

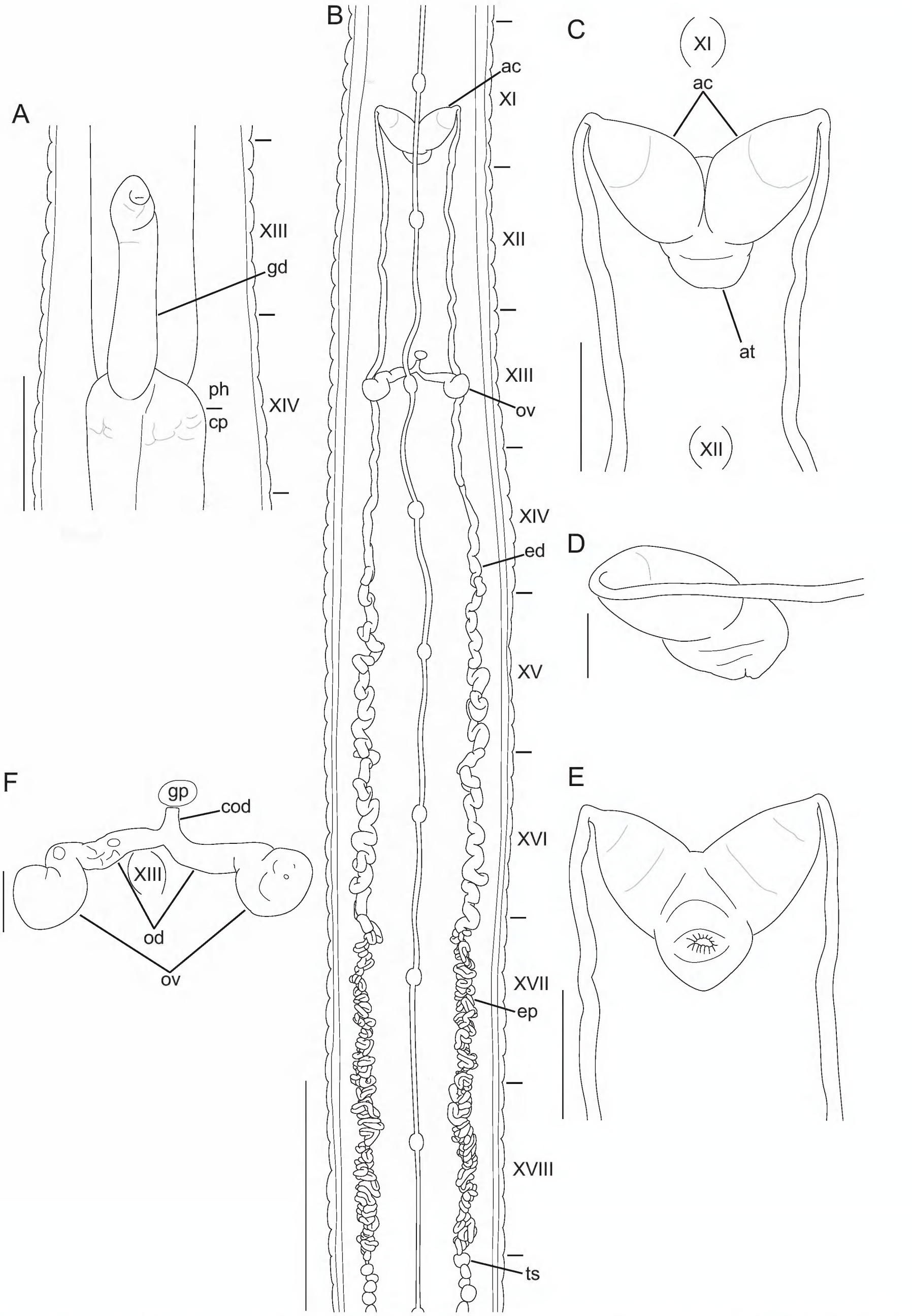

Figure 3. *Orobdella ibukifukuyamai* sp. nov., holotype, KUZ Z4031. **A.** Ventral view of gastroporal duct; **B.** Dorsal view of reproductive system, including ventral nervous system; **C.** Dorsal view of male atrium including positions of ganglia XI and XII; **D.** Left lateral view of male atrium; **E.** Ventral view of male atrium; **F.** Dorsal view of female reproductive system, including position of ganglion XIII. Scale bars: 5 mm (**A**); 10 mm (**B**); 2 mm (**C, E**); 1 mm (**D, F**).

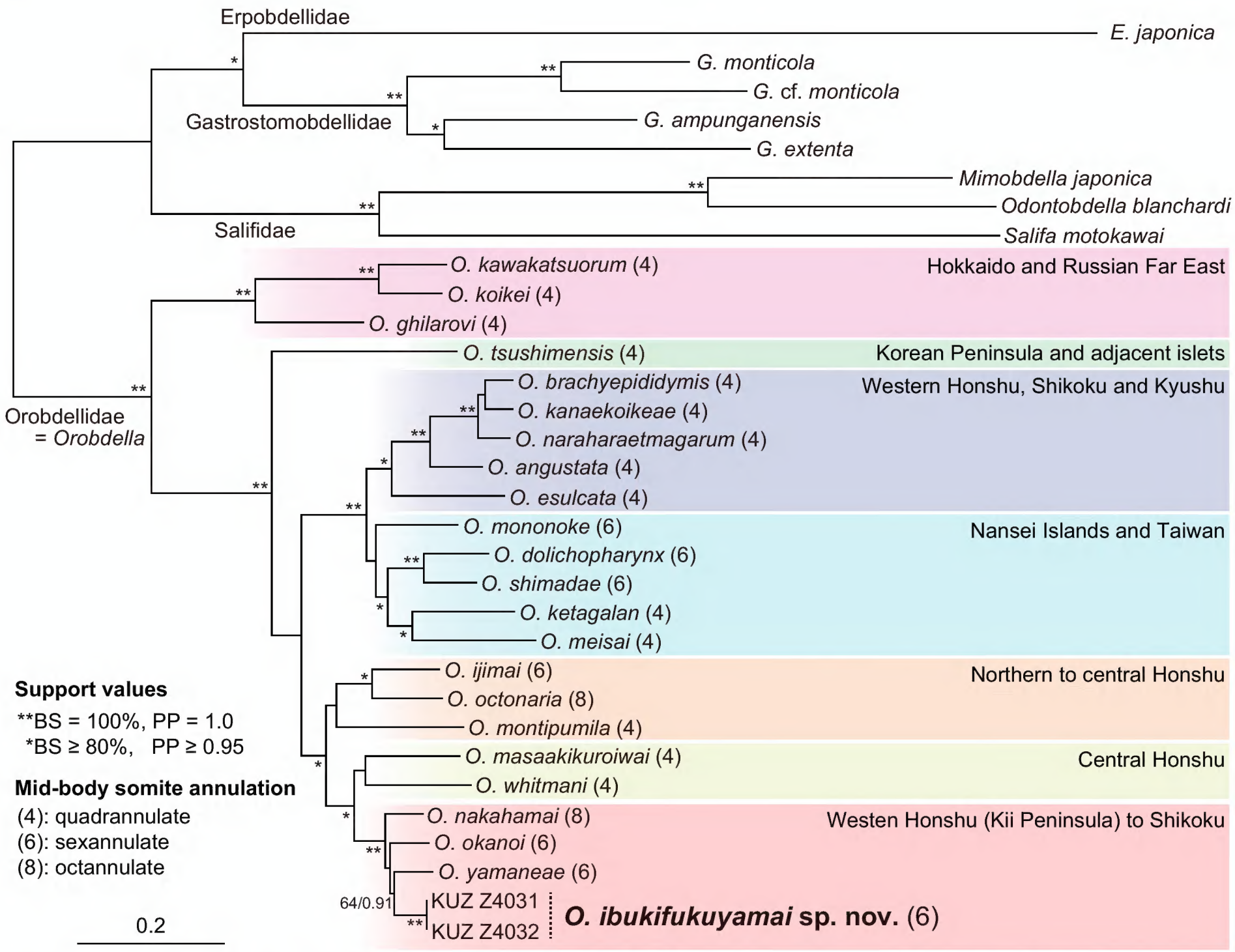

Figure 4. Bayesian inference tree for 8090 bp of nuclear 18S rRNA, 28S rRNA and H3, and mitochondrial COI, $tRNA^{Cys}$, $tRNA^{Met}$, 12S rRNA, $tRNA^{Val}$, 16S rRNA, $tRNA^{Leu}$ and ND1 markers. Numbers on nodes indicate bootstrap (BS) values for maximum likelihood ≥ 60% and Bayesian posterior probabilities (PP) ≥ 0.90. Double asterisks denote the node with BS = 100% and PP = 1.0; single asterisk denotes the node with BS ≥ 80%, PP ≥ 0.95. Numbers in parentheses represent the mid-body somite annulation of each species. The geographical range of each group/lineage within *Orobdella* is also indicated.

Table 1. Uncorrected *p*-distances for the 1267 bp COI sequences of *O. ibukifukuyamai* sp. nov. and the closely related sexannulate species, *O. okanoi* and *O. yamaneae*. Acronym: KUZ, Zoological Collection of Kyoto University.

Species	Voucher	(1)	(2)	(3)	(4)	(5)	(6)
(1) *O. ibukifukuyamai* sp. nov.	KUZ Z4031 (holotype)						
(2) *O. ibukifukuyamai* sp. nov.	KUZ Z4032 (paratype)	0.2%					
(3) *O. okanoi*	KUZ Z1671 (holotype)	5.0%	5.0%				
(4) *O. okanoi*	KUZ Z1491	4.6%	4.4%	2.5%			
(5) *O. yamaneae*	KUZ Z1678 (holotype)	4.6%	4.7%	6.0%	5.8%		
(6) *O. yamaneae*	KUZ Z1358 (paratype)	4.7%	4.9%	5.9%	5.9%	0.4%	

Nakano, 2016 and octannulate *O. nakahamai* Nakano, 2016. The monophyly of these four species was fully supported (BS = 100%, PP = 1.0). Within this clade, the monophyly of *O. ibukifukuyamai* and *O. yamaneae* was implied (BS = 64%, PP = 0.91); nonetheless, the present phylogenies failed to resolve robust relationships among the four species.

The pairwise COI uncorrected *p*-distance between the holotype and paratype of *O. ibukifukuyamai* was 0.2% (2/1267 nucleotides were polymorphic). The COI genetic divergence between *O. ibukifukuyamai* and the closely related *O. yamaneae* was 4.6–4.9% (mean = 4.7%); that between *O. ibukifukuyamai* and *O. okanoi* was 4.4–5.0% (mean = 4.7%) (Table 1). The COI genetic distance between *O. okanoi* and *O. yamaneae* was 5.8–6.0% (mean = 5.9%).

Remarks. The morphological features (see Nakano 2016) as well as molecular phylogenetic position of *O. ibukifukuyamai* clearly elucidated that this new species belongs to the genus *Orobdella*. Only two specimens of the new species could be examined in this study, but nonetheless, their morphological characteristics and DNA sequences successfully ensured the distinct species status of *O. ibukifukuyamai* within *Orobdella*.

The new species is distinguishable from the six sexannulate congeners, *O. dolichopharynx* Nakano, 2011,

Table 2. Comparisons of morphological characters between *Orobdella ibukifukuyamai* sp. nov. and six sexannulate congeners plus the octannulate *O. nakahamai*.

Species	Dorsal color	Somite VII	Somite VIII	Annuli between gonopores	Pharynx length	Gastroporal duct	Epididymides	Pre-atrial loop	Atrial cornua
Orobdella ibukifukuyamai sp. nov.	reddish brown	quinquannulate	sexannulate	1/2 + 8	to XIV	thick tubular	XVI to XVIII (11–12 annuli)	absent	ovate
Orobdella okanoi	reddish	quinquannulate	sexannulate	8 + 1/2	to XIV	bulbous	XV to XVII (8–11 annuli)	absent	ellipsoid
Orobdella yamaneae	purplish	quinquannulate	sexannulate	1/2 + 7 + 1/2	to XIV	bulbous	XVI to XVIII (8–11 annuli)	extending to anterior of XI c9	ovate
Orobdella dolichopharynx	yellowish green	quadrannulate	quinquannulate	8	to XVI	rudimentary tubular, reaching to XVI	absent	extending to ganglion XI	absent
Orobdella ijimai	yellowish green	quadrannulate	sexannulate	1/2 + 7 + 1/2	to XIV	bulbous	XVI to XIX (12–17 annuli)	absent	ellipsoid
Orobdella mononoke	anterior and posterior parts grayish purple, mid-body amber	quadrannulate	sexannulate	8 + 1/2	to XIV	tubular, but bulbous at junction with crop	XV to XIX (20–22 annuli)	absent	ovate
Orobdella shimadae	yellowish green	triannulate	quinquannulate	9	to XVI	rudimentary tubular, reaching to XV	absent	extending to ganglion XI	absent
Orobdella nakahamai	purplish	quinquannulate	dorsally septannulate, ventrally octannulate	1/2 + 11	to XIV	bulbous	XV to XVII (12–13 annuli)	absent	ovate

O. ijimai Oka, 1895, *O. mononoke* Nakano, 2012, *O. okanoi*, *O. shimadae* Nakano, 2011, and *O. yamaneae* by the following combination of characteristics (Nakano 2011, 2012, 2016; Table 2): dorsal surface reddish in life, quinquannulate somite VII, sexannulate somite VIII, 1/2 + 8 annuli between gonopores, pharynx reaching to somite XIV, thick tubular gastroporal duct, epididymides in somites XVI–XVIII that occupy 11–12 annuli, and developed ovate atrial cornua without pre-atrial loop. *Orobdella ibukifukuyamai* can be distinguished from the four sexannulate species, i.e., *O. dolichopharynx*, *O. ijimai*, *O. mononoke* and *O. shimadae* by its somite VII that is quinquannulate (quadrannulate in *O. dolichopharynx*, *O. ijimai* and *O. mononoke*, and triannulate in *O. shimadae*). Additionally, *O. ibukifukuyamai* is distinguishable from *O. dolichopharynx* and *O. shimadae* by the developed gastroporal duct and atrial cornua (both organs are rudimentary in *O. dolichopharynx* and *O. shimadae*). *Orobdella ibukifukuyamai*, *O. okanoi* and *O. yamaneae* shares several morphological features, i.e., a large body size reaching ~15 cm, a similar annulation pattern of somites VII and VIII, and a similar position of epididymides. However, *O. ibukifukuyamai* can be discriminated from *O. okanoi* and *O. yamaneae* by the reddish brown dorsal surface and ocher lateral surface, the possession of 1/2 + 8 annuli between the gonopores, and the ovate atrial cornua without pre-atrial loops (in *O. okanoi*, uniformly reddish dorsal to lateral surface, 1/2 + 8 annuli between gonopores, and ellipsoidal atrial cornua without pre-atrial loops; in *O. yamaneae*, uniformly purplish dorsal to lateral surface, 1/2 + 7 + 1/2 annuli between gonopores, and ovate atrial cornua each with slight pre-atrial loop). *Orobdella ibukifukuyamai* can be clearly distinguished from the quadrannulate and octannulate species, in which the closely related *O. nakahamai* is included (see Table 2), by its sexannulate mid-body somites.

The present molecular phylogenies recovered the close relationships among the three morphologically-similar sexannulate species, *O. ibukifukuyamai*, *O. okanoi*, and *O. yamaneae*. The calculated interspecific COI distances between *O. ibukifukuyamai* and *O. okanoi* (4.4–5.0%) was almost equal to those between *O. ibukifukuyamai* and *O. yamaneae* (4.6–4.9%). A previous study indicated that the COI divergences between the two closest species *O. kanaekoikeae* Nakano, 2017b and *O. brachyepididymis* Nakano, 2016, which are morphologically well-differentiated from each other, were 3.6–4.6% (Nakano 2017b). Therefore, the present distance values can support the distinctive taxonomic status of *O. ibukifukuyamai* within the lineage that consists of the sexannulate *Orobdella* species. Additionally, the present phylogenies recovered the monophyly of the octannulate *O. nakahamai* and the three sexannulate species; the monophyly of *O. nakahamai*, *O. okanoi* and *O. yamaneae* has been already shown by previous studies (e.g., Nakano 2021). Given the fact that *O. nakahamai* possesses the octannulate mid-body somites denoted as b1 = b2 = b3 = b4 = c9 = c10 = c11 = c12, and *O. ibukifukuyamai* bears the sexannulate mid-body somites, of which annuli a2 and b5 are, respectively, subdivided dorsally, defined as b1 = b2 < a2 (b3 = b4 dorsally) = b5 (c9 = c10 dorsally) > c11 = c12, the new species may exhibit annulation that is the transitional state (= secondarily octannulate dorsally) between sexannulate and octannulate *Orobdella* species.

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Supplementary material 1

Table S1. Samples used for phylogenetic analyses

Authors: Takafumi Nakano

Data type: sequence accession numbers (excel file)

Explanation note: Samples used for phylogenetic analyses. The information on the vouchers is accompanied by the International Nucleotide Sequence Databases (INSD) accession numbers. Sequences marked with an asterisk (*) were obtained for the first time in the present study..

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